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Arginine Metabolism in Developing Soybean Cotyledons

III. Utilization[1]

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ABSTRACT

Tracerkinetic experiments were performed using L-[guanidino-^{14}C]arginine, L-[U-^{14}C]arginine, L-[ureido-^{14}C]citrulline, and L-[1-^{14}C]ornithine to investigate arginine utilization in developing cotyledons of *Glycine max* (L.) Merrill. Excised cotyledons were injected with carrier-free ^{14}C compounds and incubated in sealed vials containing a CO_2 trap. The free and protein amino acids were analyzed using high performance liquid chromatography and arginine-specific enzyme-linked assays. After 4 hours, 75% and 90% of the ^{14}C metabolized from [guanidino-^{14}C]arginine and [U-^{14}C]arginine, respectively, was in protein arginine. The net protein arginine accumulation rate, calculated from the depletion of nitrogenous solutes in the cotyledon during incubation, was 17 nanomoles per cotyledon per hour. The data indicated that arginine was also catabolized by the arginase-urease reactions at a rate of 5.5 nanomoles per cotyledon per hour. Between 2 and 4 hours $^{14}CO_2$ was also evolved from carbons other than C-6 of arginine at a rate of 11.0 nanomoles per cotyledon per hour. It is suggested that this extra $^{14}CO_2$ was evolved during the catabolism of ornithine-derived glutamate; ^{14}C-ornithine was a product of the arginase reaction. A model for the estimated fluxes associated with arginine utilization in developing soybean cotyledons is presented.

The maximum specific radioactivity ratios between arginine in newly synthesized protein and total free arginine in the ^{14}C-citrulline and ^{14}C-ornithine experiments indicated that only 3% of the free arginine was in the protein precursor pool, and that argininosuccinate and citrulline were present in multiple pools.

Nitrogen budgets for developing cowpeas (11) and soybeans (8) suggest that the legume cotyledon plays a major role in the provision of amino acids for the synthesis of seed protein. A comparison between the N composition of soybean seedcoat exudate and cotyledonary protein suggests that 35% of the N in exudate is directly incorporated into protein, whereas 65% of the N undergoes transformation before incorporation (8). This comparison assumes that amino acids delivered to the cotyledon in deficit amounts are not metabolized. In addition, biochemical pathways not directly related to protein amino acid biosynthesis, and the existence of multiple cellular amino acid pools are not considered. In-depth metabolic studies of all seed amino acids are required to understand fully the processing of incoming N.

Arginine is the predominant form of amino acid N in soybean cotyledons, constituting 18% of the total protein N and 14% to 62% of the free pool N during seed development (8). Micallef and Shelp (8) estimated that 72% of the cotyledon's Arg requirement for protein synthesis is satisfied by *in situ* biosynthesis, and, correspondingly, ^{14}C tracer studies have demonstrated that this amino acid is actively synthesized in developing cotyledons (9). The remaining Arg is supplied by the vascular system.

In this study, ^{14}C tracer experiments involving L-[guanidino-^{14}C]Arg and L-[U-^{14}C]Arg indicated that exogenously supplied Arg was incorporated into seed protein and was metabolized via the arginase reaction and additional degradatory routes. In ^{14}C tracer experiments that used L-[ureido-^{14}C]Cit[2] and L-[1-^{14}C]Orn, which are intermediates of Arg biosynthesis (9), the specific radioactivity of the Arg incorporated into protein was compared to that in the free pool; this information indicated the existence of multiple cellular pools of Arg and other pathway intermediates. In addition, the depletion of free pool N compounds provided an estimate of the rate of Arg incorporated into protein, and, consequently, permitted estimates of the fluxes through the various pathways associated with Arg utilization.

MATERIALS AND METHODS

Soybean plants were grown, and developing cotyledons were chosen from seeds at 23 DAA as previously described (8).

All L-^{14}C-amino acids were obtained from Du Pont-New England Nuclear. The shipping medium was replaced by 50 mM Hepes (pH 8.0), and the ^{14}C solution was injected into intact excised cotyledons, which were incubated at 30°C in a closed 34-mL serum bottle containing a CO_2 trap, as described by Micallef and Shelp (8).

Tissue was ground in sulfosalicylic acid (30 mg/mL), and the supernatant was collected; the protein was extracted from the pellet and hydrolyzed in 6 N HCl (8). The composition and ^{14}C content of the amino acids present in the soluble fraction were determined by reverse-phase HPLC after *ortho*-phthalaldehyde derivatization (10). The techniques for the determination of the intramolecular position (C-1 or C-6) of

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[2] Abbreviations: Cit, citrulline; Abu, 4-aminobutyric acid; Orn, ornithine; ArgS, argininosuccinate; DAA, days after anthesis.

^{14}C in free and protein Arg have been reported (8). Ammonium was determined using the microdiffusion-nesslerization technique previously described by Shelp *et al.* (14). Nitrate was determined colorimetrically following derivatization with salicylic acid as previously described (2); background values were determined by mixing equivalent samples with derivatizing agent lacking salicylic acid. Ureides (allantoin + allantoic acid) were determined using the method described by Vogels and Van Der Drift (18).

Statistical treatment of the data was performed using the Student's *t* test procedure (16). Significance indicates significance at the 5% level unless otherwise stated.

RESULTS

Metabolism of ^{14}C-Arginine

In the first experiment, the metabolism of [guanidino-^{14}C]Arg and [U-^{14}C]Arg was investigated (Fig. 1). For both compounds, the patterns of free Arg depletion and protein accumulation were similar. There was a 40- to 60-min lag in the incorporation of ^{14}C into protein, but within 4 h 75% to 90% of the Arg removed from the free pool was recovered as protein Arg. Three to eleven percent of total ^{14}C was recovered as CO_2 after 4 h. With [guanidino-^{14}C]Arg, ^{14}C-Arg accumulation in protein and $^{14}CO_2$ evolution between 1 to 4 h exhibited a 3.5:1 ratio. After 4 h in the [U-^{14}C]Arg feeding, 6800 ± 628 dpm (mean ± SE) and 3900 ± 180 dpm of ^{14}C was recovered in Orn and Abu respectively, and there was no significant difference between the HPLC and enzymatic determinations of ^{14}C in free arginine. With both ^{14}C-labeled sources of Arg, all of the ^{14}C recovered in protein was found as Arg.

Evolution of $^{14}CO_2$ from each ^{14}C-labeled compound is shown in detail in Figure 1B. The difference between [U-^{14}C]Arg and [guanidino-^{14}C]Arg represents the loss of label from carbons other than C-6 because the radioactivity injected resulted in identical molar levels and specific radioactivities of C-6 for the two species. Essentially all of the $^{14}CO_2$ recovered up to 2 h was released from C-6 of Arg, and between 2 and 4 h the level of $^{14}CO_2$ evolved from [U-^{14}C]Arg was 3 times the level from [guanidino-^{14}C]Arg.

Rate of Protein Arginine Incorporation

Changes in the free pool N of the soybean coytledon incubated for 3 h in the absence of exogenous N are shown in Figure 2. After 3 h, major losses on a percentage basis occurred with Abu (69%), Glu (60%), Tyr (47%), and Ser (43%). Levels of Arg biosynthetic intermediates and the remaining amino acids in the "Others" category did not change significantly. Ammonium was the only N form to increase significantly. The total free pool N (11,597 nmol·cotyledon^{-1} at time zero) declined by about 10% (1151 nmol·cotyledon^{-1}) after 3 h. Assuming that all the lost N was incorporated into protein, the net protein Arg accumulation rate was 17 nmol·cotyledon^{-1}·h^{-1} (384 nmol of N·cotyledon^{-1}·h^{-1} × [18% Arg N/total protein N] [8]/[4 mol N/mol Arg]).

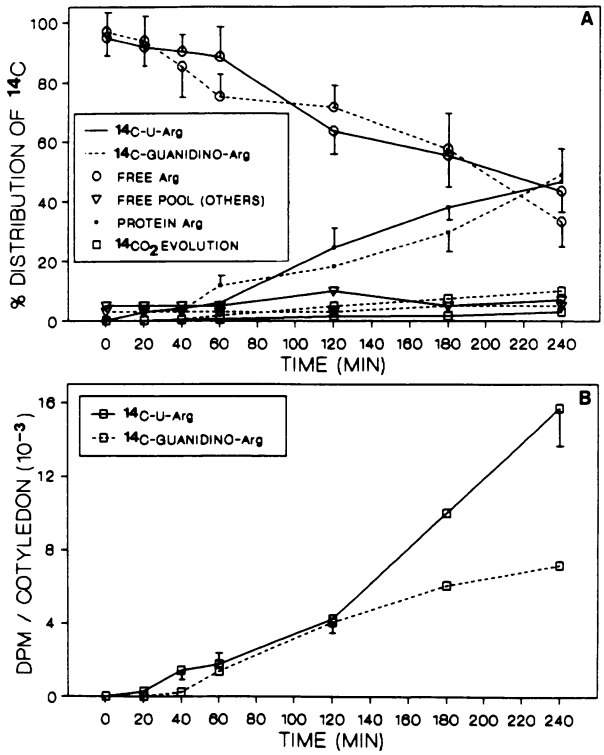

Figure 1. A, Distribution of ^{14}C with time among products of [guanidino-^{14}C]Arg and [U-^{14}C]Arg metabolism by an excised soybean cotyledon (23 DAA). Two microliters of a solution containing 50 mM Hepes (pH 8.0) and carrier-free [guanidino-^{14}C]Arg (0.04 μCi) or [U-^{14}C]Arg (0.24 μCi) was injected per cotyledon. Total percent recovery for each time point was 93% to 100% of the total ^{14}C injected at time zero. Total ^{14}C in free and protein Arg was determined using the arginase-urease assay; the free and protein Arg values obtained for the [U-^{14}C]Arg feeding were multiplied by 6 to account for total ^{14}C in the uniformly labeled molecule. B, Time course of $^{14}CO_2$ production from [guanidino-^{14}C]Arg and [U-^{14}C]Arg metabolism by an excised cotyledon (23 DAA). Data are from the experiment described in A. The data are corrected for $^{14}CO_2$ evolution resulting from ^{14}C-urea impurities present in [U-^{14}C]Arg (0.16% of total ^{14}C) and [guanidino-^{14}C]Arg (0.19% of total ^{14}C) as previously described in ref. 9. Each point is the mean of three injected cotyledons. Bars represent ± 1 SE and are not presented when smaller than the symbol.

Specific Radioactivity Determinations

With information about the rate of Arg incorporation into protein (including turnover, see "Discussion") it was possible to calculate the specific radioactivity of new-protein Arg when ^{14}C was provided as ^{14}C-Cit or ^{14}C-Orn by dividing the ^{14}C appearing in protein Arg (dpm) by the Arg incorporated into protein (μmol) in the same time interval (Fig. 3). The absolute levels of ^{14}C in the products of ^{14}C-Cit and ^{14}C-Orn metabolism have been reported (9). The specific radioactivity of new-protein Arg was, respectively, 16 to 30 and 20 to 46 times greater than the total Arg in the free pool. In each case, the specific radioactivity ratio decreased with time. The maximum specific radioactivity of new-protein Arg (6.4 × 10^6 dpm μmol^{-1} at 2 h) (Fig. 3A) was 3.2 times greater than the maximum specific radioactivity of ArgS, which occurred at 45 min. It was also greater than the specific radioactivity of

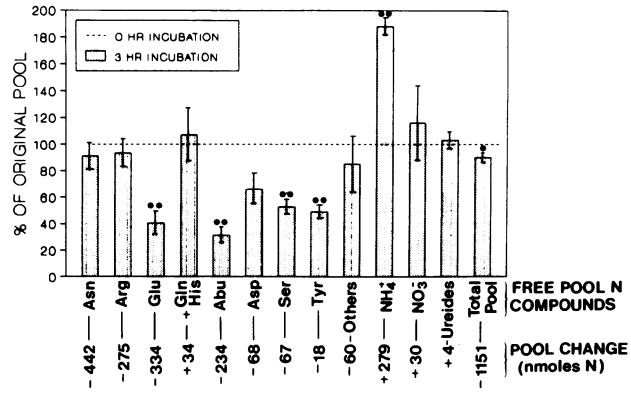

Figure 2. Changes in the free pool nitrogen from an excised soybean cotyledon (23 DAA) incubated for 3 h at 30°C. The "Others" category includes Ala, ArgS, Cit, Gly, Ile, Leu, Lys, Met, Orn, Phe, Thr, Trp, Val, and unknowns. The calculation of nitrogen contained in the unknowns was as described previously (8). Values are the mean of five cotyledons and the vertical bars represent ± 1 SE. Asterisks indicate a significant difference from the zero hour control at a 5% (**) and 10% (*) level. Significance was determined using the raw data.

Cit for most of the time course (Fig. 3B). Note that the specific radioactivity of ArgS may be an underestimate because the ^{14}C in ArgS was not corrected for *ortho*-phthalaldehyde-derivative instability (10).

In both the ^{14}C-Cit and ^{14}C-Orn feedings, there was a greater initial lag in specific radioactivity for new-protein Arg than either ArgS or Cit, respectively.

DISCUSSION

Arginine Incorporation into Cotyledonary Protein

The net rate of Arg incorporation into protein (17 nmol·cotyledon^{-1}·h^{-1}), as determined from the depletion of nitrogenous solutes (Fig. 2), corresponded closely to that found *in vivo* (13 nmol·cotyledon^{-1}·h^{-1}) (8). The rate of protein Arg turnover was not determined experimentally but was estimated using the following considerations: (a) Madison *et al.* (7), using cultured soybean cotyledons, determined that the 11S and 7S storage proteins do not turn over and that the 2S fraction has a half-life of 17 d. (b) Soybean cotyledons used in the present study (23 DAA) contain 3536 nmol of protein Arg per cotyledon (8). (c) Holowach *et al.* (4) showed that the 11S + 7S proteins constitute 50% of the total protein at 23 DAA; thus, it was assumed that the 2S fraction constituted the remaining 50%. In addition, these workers showed that the molar percentage of Arg in the 2S fraction is 50% of the molar percentage in the 11S + 7S fractions. Therefore, the Arg in the 2S fraction would be 1178 nmol (3536 nmol/3). (d) Degradation of the 2S protein is defined by the equation $A = 1178e^{-(0.693/[17\ d \times 24\ h/d])t}$, where A = final Arg content (nmol) and t = time (h). Thus, the protein Arg turnover rate would equal 2 nmol·cotyledon^{-1}·h^{-1}. With this information, the gross rate of Arg incorporation into protein is estimated as 19 nmol·cotyledon^{-1}·h^{-1} (Fig. 4).

The ^{14}C-labeling patterns obtained in the [guanidino-^{14}C]Arg experiment (Fig. 1) and in a previous study using [ureido-

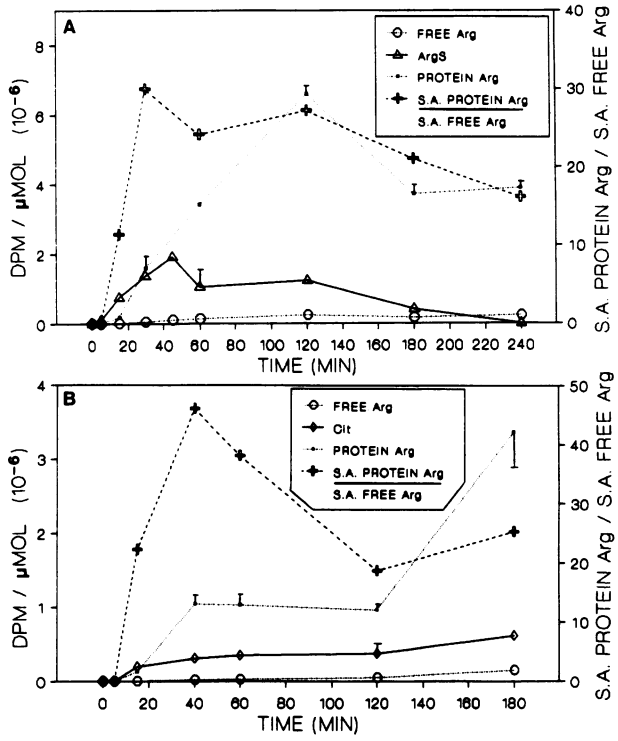

Figure 3. A, Specific radioactivity (S.A.) of free Arg, ArgS, and new-protein Arg after injection of [ureido-^{14}C]Cit into an excised soybean cotyledon (23 DAA). New-protein Arg data are plotted at the end of each sampling interval, and points representing free amino acids describe the specific radioactivity at the sampling times indicated on the abscissa. The ^{14}C in protein Arg was determined using the arginase-urease assay, and the ^{14}C in free amino acids was determined by HPLC. All of the ^{14}C in protein could be accounted for in C-6 of Arg. A protein Arg accumulation rate of 19 nmol·cotyledon^{-1}·h^{-1} (17 nmol·cotyledon^{-1}·h^{-1} net accumulation + 2 nmol·cotyledon^{-1}·h^{-1} turnover) was used. B, Specific radioactivity of free Arg, Cit, and new-protein Arg after injection of [1-^{14}C]Orn into an excised soybean cotyledons (23 DAA). The ^{14}C in protein Arg was determined using the arginine decarboxylase assay; all of the ^{14}C in protein could be accounted for in C-1 of Arg. All other determinations are as described in A. Points representing free pool components are the mean of two cotyledons, and protein determinations are the mean of three cotyledons. Vertical bars represent ± 1 SE and are not presented when smaller than the symbol.

^{14}C]Cit (9) suggest that exogenously and biosynthetically derived Arg are similiarly utilized. With both ^{14}C substrates, there was a lag in the incorporation of ^{14}C into protein and in the evolution of $^{14}CO_2$, and between 1 and 4 h there was a 3.5 to 1 ratio between ^{14}C-Arg incorporated into protein and ^{14}C released as $^{14}CO_2$. Since only one carbon was labeled in each substrate, there was a 1:1 ratio between labeled carbons and labeled molecules, resulting in a 1:1 ratio between ^{14}C% and molar%. Further considerations suggesting that the two sources of Arg could be similarily utilized are: (a) the last two reactions in Arg biosynthesis are believed to be cytosolic, (b) Arg entering the cells would initially be cytosolic, and (c) protein synthesis occurs in the cytosol (13).

We have previously calculated that 72% of the Arg in cotyledonary protein is supplied by *in situ* biosynthesis in

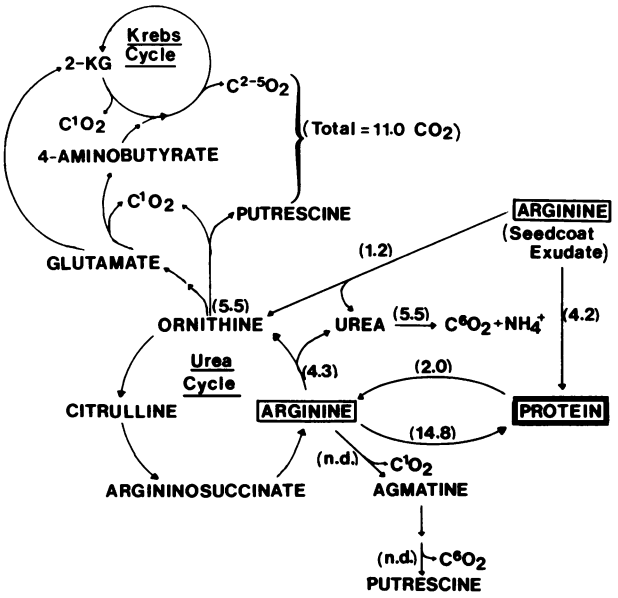

Figure 4. Fluxes associated with Arg utilization in a developing soybean cotyledon 23 DAA. The relationships were determined using data from the present study and refs. 8 and 9. Values in parentheses are in nmol·cotyledon^{-1}·h^{-1}; n.d., not detected; 2-KG, 2-oxoglutarate. The numbers associated with the CO_2 molecules represent the Arg carbon from which the CO_2 is derived.

soybeans, and that the remaining 28% is supplied by the vascular system (8). However, these calculations assume that the vascular-derived Arg is only used for protein synthesis. The present study showed that 22% of exogenously derived Arg is metabolized via the arginase reaction, and that 78% is used for protein synthesis. These data allow a recalculation of the contributions by the two Arg sources to protein synthesis. Therefore, vascular-derived Arg would directly supply only 22% (28%−[28% × 22%]) of that required for protein synthesis (Fig. 4). These calculations demonstrate that the cotyledon plays a major role in the provision of protein Arg, and this conclusion is confirmed by our finding that Arg is actively synthesized in developing soybean cotyledons (9). Since 18% of the N in cotyledonary protein is contained in Arg (8), the Arg biosynthetic pathway in the cotyledon represents a major sink for incoming N.

Arginine Catabolism

The evolution of $^{14}CO_2$ from [guanidino-^{14}C]Arg (Fig. 1) and the recovery of ^{14}C-Orn from [U-^{14}C]Arg are consistent with the breakdown of Arg to Orn and urea by arginase (EC 3.5.3.1) and subsequent hydrolysis of urea to CO_2 and NH_3 by urease (EC 3.5.1.5.). Arginase activity has not been studied in developing soybean seeds, but DeRuiter and Kolloffel (3) have detected arginase activity in developing pea seeds that was equivalent to that in germinating seeds. Urease activity has been found in developing soybean seeds (9). Our studies also show that, when [ureido-^{14}C]Cit is fed to developing seeds, ^{14}C is evolved as CO_2 (9); it is likely derived from ^{14}C-labeled Arg via the arginase and urease reactions (Fig. 4).

The results, however, indicated that multiple degradatory reactions exist for the Arg skeleton in cotyledonary tissue (Fig. 4). The route to putrescine via agmatine (15) is probably not involved since the $^{14}CO_2$ release from [U-^{14}C]Arg and [guanidino-^{14}C]Arg was similar during the first 2 h (Fig. 1). Other possibilities likely involve the metabolism of Orn (Fig. 4), which apparently was not recycled in the urea cycle; ^{14}C label from [U-^{14}C] Arg metabolism was not found in Cit, and the ^{14}C recovered in free and protein Arg was uniformly distributed. Use of arginase-derived Orn in catabolic reactions rather than recycling via the urea cycle is consistent with compartmentation of these reactions in plants as proposed by Shargool *et al.* (13).

The rate of $^{14}CO_2$ evolution between 2 and 4 h from [U-^{14}C]Arg was 3 times that from [guanidino-^{14}C]Arg (Fig. 1B). Therefore, CO_2 release from carbons other than C-6 was 58% (29% [the rate of arginase-urease reactions relative to protein Arg accumulation] × 2) of the protein Arg accumulation rate. If there was a molar ratio of 1:1 between $^{14}CO_2$ evolution and the utilization of a labeled compound, as would be expected with the direct decarboxylation of Orn (1), the rate of putrescine biosynthesis would equal 11.0 nmol·cotyledon^{-1}·h^{-1} (19 nmol·cotyledon^{-1}·h^{-1} × 0.58) between 2 and 4 h. Calculations using data from Lin *et al.* (6) indicate that the net rate of polyamine accumulation in cotyledons at 23 DAA is 0.4 nmol·cotyledon^{-1}·h^{-1}, which is only about 3% of the flux calculated above.

Ornithine also could be transaminated to glutamate 5-semialdehyde (3), and converted to Glu, which is decarboxylated to Abu by glutamate decarboxylase (EC 4.1.1.15) (8). In the present study, no ^{14}C-Glu was detected in the free pool, but ^{14}C was recovered in Abu. Further catabolism of Abu or oxoglutarate, the product of Glu deamination, would involve Krebs cycle activity and the production of CO_2 (Fig. 4).

Multiple Pools of Arginine and Arginine Pathway Intermediates

The specific radioactivity of intermediates of Arg biosynthesis provides evidence for the existence of multiple pools in the cotyledon (Fig. 3). The level of a precursor in a metabolic pool can be estimated from the maximum ratio between the specific radioactivity of a product and its precursor (5). In the ^{14}C-Cit and ^{14}C-Orn experiments (Fig. 3), the maximum specific radioactivity ratio between new-protein Arg and total free Arg was 30:1 and 46:1, respectively, giving an average ratio of 38:1. These ratios indicate that only 3% (30 nmol) of free Arg was in the protein precursor pool. It is possible that the remaining Arg (970 nmol) was contained in vacuoles. Indeed, a fractionation study with apple cotyledons demonstrated that 95% of the total free Arg is vacuolar (19), and electron microscopy has shown that large vacuoles are present in soybean cotyledon cells at mid-podfill (13). The ratio between the specific radioactivity of new-protein Arg and of total free Arg decreased with time for both ^{14}C-labeled substrates (Fig. 3), suggesting that a portion of the newly synthesized Arg had become sequestered in the vacuoles, thereby excluded from the protein precursor pool. The same results have been obtained with *Neurospora*, which sequesters 95% to 99% of its free Arg in vacuoles (5).

The specific radioactivity data (Fig. 3) also supports the

existence of multiple pools of Cit and ArgS in cotyledonary tissue, because newly synthesized Arg should not have a specific radioactivity greater than its precursors in a one-compartment system (17). For Cit, these pools could represent a plastid pool associated with ornithine carbamoyltransferase (EC 2.1.3.3.) and a cytosolic pool associated with ArgS biosynthesis (13). However, it is uncertain where a second pool of ArgS would exist in addition to the cytosolic pool. In *Neurospora*, ^{14}C tracer work also provides evidence that part of the Cit and ArgS pools are sequestered from the protein precursor pool (5), but that study did not separate Cit and ArgS.

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